PATENT
Customer No. 22,852
Attorney Docket No. 08702.0086-00000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
MAJUMDAR et al.	Group Art Unit: 1646
Application No.: 10/078,808	Examiner: J.L. Andres
Filed: February 19, 2002	
For: CHONDROGENIC POTENTIAL OF HUMAN BONE MARROW-DERIVED CD105+ CELLS BY BMP	Confirmation No.: 7146

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION OF MANAS MAJUMDAR
UNDER 37 C.F.R. § 1.131

1. I, Manas Majumdar, declare that I am one of the named applicants of the above-identified application and am one of the co-inventors of the subject matter described and claimed therein.

2. I have been advised that the United States Patent Office has rejected the claims of the above-identified application as obvious in view of International Patent Publication WO/29552.

3. I make this declaration to establish that prior to May 25, 2000, Elisabeth Morris and I completed in this country the invention described and claimed in the above-identified application.

4. To establish that the claimed invention was reduced to practice prior to May 25, 2000, I attach documents that detail the work Elisabeth Morris and I performed or directed to be performed in connection with the invention (Exhibits A-F). All of these documents were prepared prior to May 25, 2000. However, specific dates have been redacted.

5. As Exhibit A, I attach a true and accurate copy of page 59 of Genetics Institute Laboratory Notebook No. 6364, prepared and signed by me prior to May 25, 2000. Page 59 documents the isolation of CD105+ cells from bone marrow by a two-step process. The cell isolation protocol is described in the top third of the page under "Isolation of Cells" and includes a 45 minute incubation with anti-CD105 antibody and a subsequent 20 minute incubation with the secondary antibody, rat anti-mouse IgG1. The lower two-thirds of the page describe analysis by flow cytometry of the input and output of the cell isolation protocol. "Summary of Flow Cytometry" shows enrichment of CD105+ cells in the isolated population (14% CD105+ by flow cytometry) relative to the starting mononuclear cell ("MNC") population (5%).

6. As Exhibit B, I attach a true and accurate copy of page 97 of Genetics Institute Laboratory Notebook No. 6364, prepared and signed by me prior to May 25, 2000. Page 97 documents the treatment of a composition of CD105+ cells in alginate culture with BMP-2. The stated purpose of this experiment is "to differentiate CD105+ cells towards chondrogenesis." In

addition to describing the alginate culture protocol, page 97 notes at the bottom of the page that the cell media were changed to media +/- BMP-2. The experiment of page 97 is entitled "MM#38: Chondrogenesis" (for "Manus Majumdar experiment # 38") and also incorporates products of experiment MM#35 (see Exhibit C).

7. As Exhibit C, I attach a true and accurate copy of page 125 of Genetics Institute Laboratory Notebook No.6364, prepared and signed by me prior to May 25, 2000. Page 125 documents the induction of Type II collagen expression, a marker of chondrogenesis, in the BMP-2-treated CD105+ cells described on page 97 (under "SOURCE OF RNA," page 125 lists experiments MM#38 and MM#35 of Exhibit B). The upper portion of page 125 describes the RT-PCR assay for Type II collagen expression that we followed. The lower portion of page 125 includes an image of the resulting gel, accompanied by notes that "Lanes: 4,6, 10, 12-14, 15, + 16 are +ive" for Type II collagen expression, and thus that "marrow stromal cells in alginate express Col II when treated with BMP-2."

8. As Exhibit D, I attach a true and accurate copy of page 21 of Genetics Institute Laboratory Notebook No. 7547, prepared and signed by my technician, Eunice Wang prior to May 25, 2000. Page 22 documents the isolation of CD105+ cells from bone marrow by a one-step process. The cell isolation protocol is described in the top half of the page under "Isolation of Cells" and includes a single 45 minute incubation with "CD105

microbeads." Under "Cell recovery," this page shows the successful isolation of 4.48 x 10^6 cells through this one-step protocol. In the top right corner, this experiment is labeled "EW#7" (see Exhibit E).

9. As Exhibit E, I attach a true and accurate copy of page 102 of Genetics Institute Laboratory Notebook No. 7547, prepared and signed by my technician, Eunice Wang prior to May 25, 2000. Page 102 documents a method of inducing chondrogenesis involving CD105+ cells isolated by the one-step process. The title at the head of page 102 reads "RT-PCR-Elisa to check Col2 expression in 2nd purified RNA from alginate culture." Thus, this page provides the results of assays for induction of Type II collagen, a marker of chondrogenic differentiation. The gel pictured in the lower right corner shows Type II collagen expression in lane 9 (the positive control) and lanes 7 and 8, corresponding to EW#7 cells (the one-step purified cells of Exhibit D) treated with BMP-2 or BMP-9, respectively.

10. As Exhibit F, I attach a true and accurate copy of Majumdar *et al.*, J. Cell. Physiol. 185:98-106 (2000). Co-inventor, Elisabeth Morris and I are co-authors of the article. As noted in the lower right corner of page 98, this manuscript was received by the journal on February 2, 2000 and accepted for publication on April 13, 2000. The article shows both one-and two-step isolations of CD105+ cells (see, for example, "Materials and Methods: Isolation and culture expansion of CD105+ cells," page 99), culture of CD105+ cells In alginate (see, for example, "Materials and Methods:

Culture of CD105+ cells in alginate," page 100), and expression of type II collagen, a marker of chondrogenesis, upon treatment of alginate-cultured CD105+ cells with TGF-β (see, for example, Results: Expression and synthesis of type II collagen by CD105+ cells," page 103).

11. I declare further that Eunice Wang, who recorded the lab notebook entries presented in Exhibits D and E, and Valerie Banks and Diane Peluso, co-authors on the article of Exhibit F, did not make inventive contributions to the reduction to practice of the claimed methods. Rather, their contributions to the subject matter of Exhibits D-F, and the claimed invention as a whole, were strictly technical and were made under my direction.

12. I further declare that I did not abandon, suppress or conceal this invention between May 25, 2000 and the filing of the above-identified application. In fact, I continued to work on the methods of the invention to optimize conditions and results prior to filing the application.

13. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Respectfully submitted,

Dated: April 5, 2005

By: Manas Kumar Majumdar
Manas Majumdar, Ph.D.

TITLE MM#20: Fractionating BM#20 PROJECT NO.

Work continued from Page

Date:
BM# 10 Poietic # C160F
Volume: 50 ml
Nucleated Cells / ml: 2.8×10^7
Total Nucleated Cells: $2.8 \times 10^7 \times 50 = 1.4 \times 10^9$
Dilution of Cells for Ficoll Separation: $1.4 \times 10^7 \div 7 \times 10^6 = 200$ ml (50 + 150 q.s.)
Mononuclear Cells (MNCs): 185×10^6
Isolation of Cells:
Cell #: 155×10^6 [Left 30×10^6 MNCs on ice)
Antibodies used and amount: CD105, 15 λ
Incubation Time and Temperature: cold room, 45'
Secondary Antibody used and amount: rat anti IgG1, 150 λ
Incubation Time and Temperature: cold room 20'
Column used: MST
Cell recovery:

$CD105^+ - 62 \times 5 \times 2 \times$

Plating:
Plate used: 12 well
Cells / ml: 50,000 cells/well/ml
Flask used: 185 cm^2
Cells / ml: 3×10^6 cells/Flask
1.T-75 for CD105+ c

Flow Cytometry:
Cells used:
Cell #:

Tube #	Sample	Primary Ab
1	MNCs	unstained
2		isotype-PE
3		CD45-PE
4		isotype-PE
5		CD14-PE
6		isotype-PE
7		CD105-PE
8		isotype-FITC
9		CD106-FITC
10		isotype PE/CD/PE (5λ+5λ)
11		CD45-PE/CD/CD14-FITC
12	$CD105^+$	
13	MNCs	isotype PE/FITC
14		CD105 PE/CD106-FITC
15	$CD105^+$	
16		
17		
18		
19		
20		

MM#20: Summary of Flow Cytometry

Sample	CD45	CD14	CD45/CD41	CD105	CD106	CD105/106
1. Unstained						
2. Isotype						
3. MNCs	91					
4. Isotype						
5. MNCs		38				
6. Isotype						
7. MNCs				9		
8. Isotype						
9. MNCs					29	
10. Isotype						
11. MNCs	36		39			
12. CD105+	11		7			
13. Isotype						
14. MNCs				5	5.2	1.7
15. CD105+				14	3.5	0.8

Replaced media
Replaced media

Work continued to Page 66

SIGNATURE Manas Majumdar DATE

DISCLOSED TO AND UNDERSTOOD BY DATE

TITLE MM#38 : Chondrogenesis PROJECT NO.

Work continued from Page

Purpose: To differentiate C3105-1 cells towards Chondrogenesis.

1. 10×10^6 cells from MM#29, Page 90
2. Centrifuged the cells washed pellet in conc of 0.15M NaCl
3. Resuspended the cells in 1.2% alginate in 0.15M NaCl.
4. Transfered the cell suspension to a syringe (5ml) with a 20 gauge needle.
5. Prepared beads by layering drops of the cell suspension over a 102mM CaCl2.
6. Washed the beads in NaCl. 3x with media
7. Placed the ~~cell~~ beads in 12-well dish at 30 beads/well. [Total of 12 wells/plate]

cells/bead = $10 \times 10^6 / 360$ = 27,000 cells/bead

1. Transfered 1 bead from each well from a plate, (total of 6 beads) placed in Bead Recovery Buffer (55mM Na-Citrate / 0.15M NaCl) in a 15ml tube.
2. Mixed by brief vortex at low speed until the beads dissolved (visually)
3. Centrifuged at 5K for 5 min, discarded Sup.
4. Resuspended in 1.0 ml trypan blue
5. Cell Count:

	1st plate	2nd plate
Viable cells	43	43
nonviable cells	27	32

6. Changed media in the wells as follows:

From Page 92 (MM# 35)

MSCM: +/- BMP-2, CHM@FBS: +/- BMP-2

CHM@ITS: +/- BMP-2

Work continued to Page 98

SIGNATURE Manas Majumdar DAT

DISCLOSED TO AND UNDERSTOOD BY Paul Ma DATE . . PROPRIETARY INFORMATION BELONGING TO GENETICS INSTITUTE

TITLE MM#38 and MM#39 PROJECT NO.

Work continued from Page 107, 109

RT-PCR PROTOCOL

SOURCE OF RNA : MM#38 MM#39 and MM#35

GENES TO BE ANALYZED Col I and Col II

RT STEP

REAGENTS	AMOUNT/RXN	# OF SAMPLES	TOTAL VOLUME
10X PCR BUF	2	35	70
MgCl2	4		140
dATP	2		70
dCTP	2		70
dGTP	2		70
dTTP	2		70
HEXAMER	1		35
RNase INHIBITOR	1		35
REVERSE TRANS	1		35
RNA	1-3 ul <or=0.5ug	2	
WATER	make up vol to 20 ul	1 x 35	35

PROGRAMME # 111 in Heidi's 9600

PCR STEP

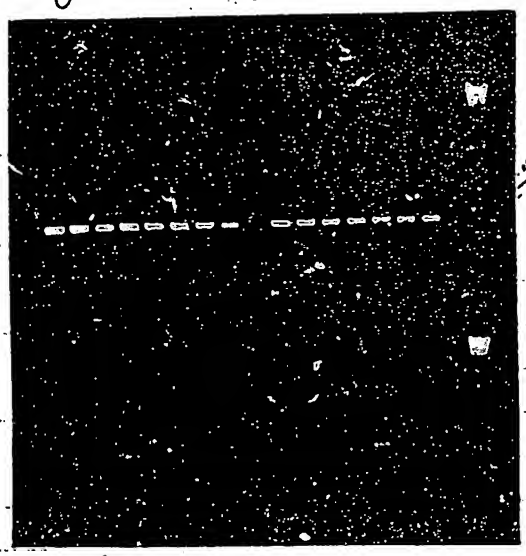
REAGENTS	AMOUNT/RXN	# OF SAMPLES	TOTAL VOLUME
10X PCR BUF	8	35	280
MgCl2	4		140
Taq Polymerase	0.5		17.5
water	65.5		2292.5
Primer 1	1		
Primer 2	1		

PROGRAMME # 115 in Heidi's 9600

Analysis: 1.2 % Agarose gel.

Col I

Col II



Results

Lanes 1 - 16 are +ive

Lanes: 4, 6, 10, 12-14, 15 & 16 are +ive.

Marrow stromal cells in alamar expresses Col II when treated with BMP-2

Work continued to Page

SIGNATURE Manas Majumdar DATE

DISCLOSED TO AND UNDERSTOOD BY DATE

No. 7547- 21

TITLE PROJECT NO. EW#7.

Work continued from Page

Date: GENETICS EW#7

BM#

Volume: 50 ml

Nucleated Cells / ml: 17×10^6

Total Nucleated Cells: 850×10^6

Dilution of Cells for Ficoll Separation: 121 ml

Mononuclear Cells (MNCs): $26/4 \times 10 \times 19.5 \times 10^4 = 126.75 \times 10^6$

Isolation of Cells:

Cell #:

CD105-microbeads amount: 400 ul

Incubation Time: 4°C, 45 min

Column used: MS (2)

Cell recovery: $36/4 \times 2 \times 4 \text{ ml} \times 10^6 = 4.48 \times 10^6$

Plating:

Plate used: 6-well

Cells / well: 100,000

Flask used: T-185 cm^2

Cells / flask: ~~0.3×10^6~~ 0.75×10^6

# of flasks: 6

change medium.

~~add 10 ml of fresh medium~~ change medium.

change the medium.

Work continued to Page

DATE

SIGNATURE

DISCLOSED TO AND UNDERSTOOD BY

DATE

nexel protos

No. 7547-102

TITLE RT-PCR-ELISA

PROJECT NO. EW#39

Work continued from Page

to check Col2 expression in sud purified RNA from alginate culture.

RT-PCR PROTOCOL

SOURCE OF RNA 2nd purified RNA from VB46, EW7 x VB26 (3.22), (-)ve, (-)ve (2/09), x dH2O (Total (12))

GENES TO BE ANALYZED β2, Col2

RT STEP REAGENTS	AMOUNT/RXN	# OF SAMPLES	TOTAL VOLUME
10X PCR BUF	1	24	24
MgCl2	2		48
dNTP	5		120
HEXAMER	0.5		12
RNase INHIBITOR	0.5		12
REVERSE TRANS	0.12		2.88
RNA	0.5		
WATER	vol to 10 ul		
PROGRAMME #			

PCR STEP REAGENTS	AMOUNT/RXN	# OF SAMPLES	TOTAL VOLUME
10X PCR BUF	4	12	48
MgCl2	2		24
Taq Polymerase	0.25		3
water	32.75		393
Primer 1	0.5		6
Primer 2	0.5		6
PROGRAMME #			

1. marker
2. —
3. VB46 - Col2
4. - BMP2
5. - " 9
6. EW7 - Col2
7. - BMP2
8. - " 9
9. ⊕ control.

3.22 RT-PCR

File: EW#39-
Instrument:dnpgly Mod:Primary:405\10 for DataVie

Molecular Dynamics Xperiment™ 1.1.0 build 22

	1 (β2)	2	3	4 (Col2)	5
A	VB46 4.3014	4.1065		VB46 0.2448	3.0973
B	3.9715	⊕ 3.6564		0.1716	⊕ 1.3407
C	4.1320	⊕ 4.2289		0.1812	⊕ 3.6382
D	EW7 3.5743	⊖ 0.2067		EW7 0.1658	⊖ 0.1738
E	3.9468	0.1304		3.1623	0.1266
F	3.5580	0.1355		2.5037	0.1225
G	VB26 4.0015	0.1288		VB26 0.1523	0.1277
H	3.5379	0.1326		1.7093	0.1265

Work continued to Page

SIGNATURE

DATE

DISCLOSED TO AND UNDERSTOOD BY

DATE

PROPRIETARY INFORMATION BELONGING TO GENETICS INSTITUTE

JOURNAL OF CELLULAR PHYSIOLOGY 185:98–106 (2000)

Isolation, Characterization, and Chondrogenic Potential of Human Bone Marrow-Derived Multipotential Stromal Cells

MANAS K. MAJUMDAR,* VALERIE BANKS, DIANE P. PELUSO, AND ELISABETH A. MORRIS

Genetics Institute Inc., Cambridge, MA

Multipotential bone marrow stromal cells have the ability to differentiate along multiple connective tissue lineages including cartilage. In this study, we developed an efficient and reproducible procedure for the isolation of stromal cells from bone marrow aspirates of normal human donors based on the expression of endoglin, a type III receptor of the transforming growth factor-beta (TGF-β) receptor family. We demonstrate that these cells have the ability of multiple lineage differentiation. Stromal cells represented 2–3% of the total mononuclear cells of the marrow. The cells displayed a fibroblastic colony formation in monolayer culture and maintained similar morphology with passage. Expression of cell surface molecules by flow cytometry displayed a stable phenotype with culture expansion. When cocultured with hematopoietic $CD34^+$ progenitor cells, stromal cells were able to maintain their ability to support hematopoiesis in vitro. Culture expanded stromal cells were placed in a 3-dimensional matrix of alginate beads and cultured in serum-free media in the presence of TGFβ-3 for chondrogenic lineage progression. Increased expression of type II collagen messenger RNA was observed in the TGFβ3 treated cultures. Immunohistochemistry performed on sections of alginate beads detected the presence of type II collagen protein. This isolation procedure for stromal cells and the establishment of the alginate culture system for chondrogenic progression will contribute to the understanding of chondrogenesis and cartilage repair. J. Cell. Physiol. 185: 98–106, 2000.

Articular cartilage damaged by trauma or disease processes has a limited spontaneous repair response due to the lack of vascular supply and a dense extracellular matrix with sparsely embedded chondrocytes. The importance of bone marrow cell components in the repair of articular cartilage has been highlighted by surgical techniques that aim to supply bone marrow-derived stromal cells to the damaged cartilage site by penetrating the underlying subchondral bone (Suh et al., 1997; O'Driscoll, 1998). These techniques ensure the migration of stromal cells to the site of repair and depend upon the surrounding environment to provide the proper stimulus leading to chondrogenic differentiation and articular surface formation. However, in the majority of the cases, repair results in fibrocartilage, not articular cartilage (Buckwalter and Mankin, 1998a; Buckwalter et al., 1990). These studies indicate our limited understanding of bone marrow stromal cells and the factors that modulate their chondrogenic differentiation.

Bone marrow consists of hematopoietic cells in close juxtaposition with the cellular components of the hematopoietic microenvironment which are a heterogeneous group of cells including stromal cells, smooth muscle cells, osteoblasts, adipocytes, and reticular endothelial cells (Bently, 1982; Tavassoli and Friedstein, 1983; Weiss, 1995). Stromal cells include mesenchymal stem and progenitor cells that have multipotential characteristics to differentiate into connective tissue lineages including osteoblasts, chondrocytes, tenocytes, adipocytes, and myocytes when placed in appropriate in vivo or in vitro environments (Haynesworth et al., 1992; Gimble et al., 1996; Ferrari et al., 1998; Mackay et al., 1998; Young et al., 1998). Stromal cells also have the ability to support hematopoiesis in vitro when cocultured with hematopoietic progenitors (Dexter et al., 1977). Therefore, stromal cells play a pivotal role both in mesengenesis and hematopoiesis.

Procedures to isolate stromal cells in vitro based on their adherent characteristic have been reported (Haynesworth et al., 1992; Kadiyala et al., 1997; Fortier et al., 1998; Pittenger et al., 1999). Haynesworth et

*Correspondence to: Manas K. Majumdar, Genetics Institute, 35 Cambridgepark Drive, Cambridge, MA 02140.
E-mail: mmajumdar@genetics.com

Received 2 February 2000; Accepted 13 April 2000

al. (1992), found that selected lots of fetal bovine serum resulted in increased adherence, proliferation, and maintenance of multipotential characteristic of stromal cells. Kadiala et al., (1997) and Pittenger et al., (1999) utilized similar procedures for their stromal cell isolation. These isolation procedures require time consuming assays performed on cells isolated by different lots of fetal bovine serum and are difficult for others to follow. Gronthos and Simmons (1995) isolated stromal cells using the antibody raised against Stro-1, a cell surface molecule transiently expressed by marrow stromal cells with yet unknown biological function. This procedure is reproducible but time consuming since it requires prior purification of the primary antibody from ascites and a secondary antibody for magnetic selection. Therefore, an efficient and reproducible stromal cell isolation procedure still remains lacking.

Factors that promote chondrogenesis or demonstrate chondrogenic effect both in vivo and in vitro include transforming growth factor beta (TGF-β), insulin-like growth factors and bone morphogenetic proteins (BMPs) (O'Driscoll SW, 1998; Sellers et al., 2000). In vitro expanded stromal cells cultured in aggregate in the presence of TGF-β acquire chondrogenic potential (Kadiyala et al., 1997; Johnstone et al., 1998; Mackay et al., 1998; Yoo et al., 1998) which suggests that stromal cells present in the bone marrow express receptors for TGF-β. Monoclonal antibodies to endoglin (CD105), a type III TGF-β receptor, have been shown to recognize cell surface molecules on stromal cells and the stromal cell line Str-5 (Robledo et al., 1996). The presence of endoglin on stromal cells and the availability of monoclonal antibody provided an opportunity to isolate these multipotential cells using the presence of a cell surface molecule of known biological function (Li et al., 1999).

The objectives of this study were to develop an efficient and reproducible procedure to isolate stromal cells from the human bone marrow based on the presence of the TGF-β receptor. This investigation continued to demonstrate the multipotential characteristic of these cells by both in vitro chondrogenic lineage progression and their ability to support hematopoiesis.

MATERIALS AND METHODS

Isolation and culture expansion of CD105$^+$ cells

Human bone marrow samples were obtained from Poietic Technologies (Gaithersburg, MD). Mononuclear cells (MNCs) were isolated according to a modification of a previously reported method (Majumdar et al., 1998). Total nucleated cells in the marrow sample was diluted to a concentration of 7×10^6 cells per ml with calcium and magnesium free phosphate-buffered saline (PBS), (Life Technologies, Gaithersburg, MD) containing 2% bovine serum albumin (BSA, Life Technologies), 0.6% sodium citrate (Life Technologies), and 1% penicillin-streptomycin (Life Technologies). Thirty to 35 ml of the diluted cell suspension was layered over 15 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 800 × g for 20 min. The mononuclear cells (MNCs) collected at the interface were recovered, counted, washed with magnetic-activated cell sorting (MACS) buffer (PBS with 0.5% BSA and 2 mM EDTA, pH 7.2). The washed cells were resuspended in MACS buffer at $2–4 \times 10^8$ cells per ml, transferred to a 5 ml tube to which 50 μl of mouse anti-human CD105 antibody (Pharmingen, San Diego, CA) was added and placed on a rotator for 1 hr at 4°C. The cells were again washed, resuspended at 2.5×10^8 per ml in MACS buffer. One quarter of a milliliter of rat anti-mouse IgG1-microbead (Miltenyi Biotec, Auburn, CA) was added and placed on a rotator for 30 min at 4°C. The cells were washed and separated on a magnetic column MS$^+$ (Miltenyi Biotec) according to the manufacturer's recommendation. The CD105$^-$ cells were collected as the column eluate, while the CD105$^+$ cells remained attached to the column. To recover the CD105$^+$ cells, the column was removed from the magnet and the cells were flushed out with MACS buffer. The above procedure was referred to as a dual antibody procedure (DAP) due to the requirement of two different antibodies. We also tested the directly conjugated mouse anti-human CD105 antibody-microbeads (Miltenyi Biotec) and compared this single antibody procedure (SAP) with DAP for CD105$^+$ cell isolation. For SAP, 1×10^8 MNCs were incubated with the 0.2 ml of anti-human CD105 antibody-microbeads for 45 min at 4°C and CD105$^+$ cells were isolated using the MS$^+$ column as described. Both the DAP and the SAP methods were evaluated with multiple human bone marrow samples.

CD105$^+$ cells isolated either by DAP or SAP were plated in 185 cm^2 Nunclon Solo flasks (Nunc Inc., Naperville, IL) at a density of $1–2 \times 10^6$ cells per flask and cultured in complete medium consisting of Alpha-MEM supplemented with 10% fetal bovine serum (FBS) and 1% antimycotic-antibiotic (Life Technologies) at 37°C in 5% CO_2 in air. Medium was changed after 48 h and thereafter every 3–4 days. At day 14, cells were detached by incubation with 0.05% trypsin-EDTA (Life Technologies) for 20–30 min at 37°C. The cells at this stage were designated primary (p0) and replated for expansion at a density of 1×10^6 cells per flask as passage 1 cells. The cells reached 90% of confluence in 6–7 days, after which they were either passaged as mentioned, used in other assays, or stored in 90% FBS and 10% dimethyl sulphoxide in liquid nitrogen for future use.

Colony forming unit-fibroblast (CFU-F) assay

MNCs, CD105$^+$, and CD105$^-$ cells were plated in 6-well plates at 1×10^5 cells per well and media were changed as described. Between 7 and 10 days, medium was removed and the cells were washed with PBS. One ml of reconstituted brilliant blue R concentrate (Sigma, St. Louis, MO) was added to each well and incubated at room temperature for 5 min. The wells were washed with tap water and the plate dried. Aggregates of 50 cells or more were scored as CFU-F.

Flow cytometry

Analysis of cell surface molecules were analyzed according to a previously reported procedure (Majumdar et al., 1998). Briefly, cells were washed in FACS buffer (2% BSA, 0.1% sodium azide in PBS) and aliquots ($2 \times 10^5–1 \times 10^6$) of cells were incubated with fluorochrome-conjugated monoclonal antibodies (Pharmingen, San Diego; Serotec, Raleigh, NC) for 20 min at 4°C. Cells were washed and the cell pellet was resuspended in FACS buffer with 1% paraformaldehyde. Nonspecific fluorescence was determined using equal aliquots of

TABLE 1. Oligonucleotide primers used for PCR amplification and ELISA detection

Oligonucleotide primers/5'-biotinylated probes	Size (bp)	Reference/Accession #
Human Beta2-microglobulin Sense: 5'-TCTGGCCTTGAGGCTATCCAGCGT-3' Antisense: 5'-GTGGTTCACACGGCAGGCATACTC-3' Probe: 5'-Biotinylated CATCCATCCGACATTGAAGTTGAC-3'	270	Majumdar et al. (1998)
Human Type II collagen Sense: 5'-TCCCAAAGGTGCTCGAGGAGA-3' Antisense: 5'-CTCACCACGATCACCCTTGAC Probe: 5'-Biotinylated GAGAGAGGATTCCCTGGCTT	400	X16468

cell preparation that were incubated with mouse isotype monoclonal antibodies. Data were collected by analyzing 10,000–50,000 events on a Becton Dickson instrument (San Jose, CA) using Cell-Quest software. For adherent cells, medium was removed, cells detached from the flasks by trypsin-EDTA treatment, recovered by centrifugation and washed in FACS buffer. Aliquots of 2×10^5 cells were incubated with antibodies and analyzed as described. The results of the cell surface studies are expressed as the mean of three donor cell populations with standard error of the mean. Flow cytometry analysis was also performed on cells detached by non-enzymatic cell dissociation buffer (Life Technologies) to analyze the effect of trypsin on cell surface molecules.

Long term bone marrow culture (LTBMC)

LTBMC was performed according to modifications of previously reported procedure (Majumdar et al., 1998). Cells from 3 donors at the end of day 14 (p0) were detached by trypsin-EDTA treatment, washed in PBS and 3×10^5 cells per well were plated in 6-well plates. At 80–90% of confluence, cells were exposed to 16 Gy irradiation, the medium was changed, and the cells cultured overnight. $CD34^+$ hematopoietic progenitor cells (Poietic Technologies) were resuspended at 5×10^4 cells per ml in LTBMC medium which consisted of Myelo Cult (Stem Cell Technologies, Vancouver, Canada), 1 μM hydrocortisone (Sigma), and 1% antimycotic-antibiotic (Life Technologies). Medium was removed from the irradiated cells and replaced with 2 ml of $CD34^+$ cell suspension per well. The cocultures were maintained at 37°C for 72 h and thereafter, at 33°C. Half the medium was replaced weekly with fresh medium for 5 weeks. Experimental controls consisted of $CD34^+$ cells without any stromal layer. After 5 weeks, both nonadherent and adherent cells were collected, resuspended in 0.3 ml of LTBMC medium and added to 2.7 ml of methylcellulose medium (Metho Cult 4435, Stem Cell Technologies). Aliquots of 1 ml of the cell suspension were plated in duplicate in 35 mm Nunc dishes (Nunc Inc.) and incubated at 37°C in 5% CO_2 for two weeks. Colonies consisting of more than 50 cells were scored and used for statistical analysis.

Culture of $CD105^+$ cells in alginate

The $CD105^+$ cells were encapsulated in alginate by modification of previously reported procedures (Mok et al., 1994; Binette et al., 1998). Cells were detached by trypsin-EDTA treatment and washed with wash buffer (0.15M NaCl, 25 mM Hepes, pH 7.0). The cells were then resuspended at a density of 25×10^6 per ml in 1.2% alginate in wash buffer. Individual beads of the cell suspension were then slowly expressed through a 20-gauge needle into a solution containing 102 mM $CaCl_2$ and 25 mM Hepes (pH 7.0). The beads were allowed to polymerize for 10 min, washed once in wash buffer, three times in complete medium, and cultured overnight in the same medium at 37°C with 5% CO_2 in air. The next day, the medium was changed to chemically defined medium (Mackay et al., 1998; Yoo et al., 1998) consisting of DMEM with high glucose, 100 nM dexamethasone (Sigma), 50 μg/ml ascorbic acid-2-phosphate (WAKO Pure Chemicals, Tokyo, Japan), 100 μg/ml of sodium pyruvate (Life Technologies), 50 μg/ml proline (Sigma), 1% ITS-Premix (Becton Dickenson, Bedford, MA) and 10 ng/ml TGF-β3 (R and D Systems, Minneapolis, MN). The medium was changed twice a week for the next 3 weeks.

RNA preparation and analysis

Culture-expanded $CD105^+$ cells from 3 donors were encapsulated in alginate beads and cultured for three weeks. At day 21 beads were transferred to cell recovery buffer (55 mM Sodium Citrate, 0.15 M NaCl and 25 mM Hepes, pH 7.0) and incubated for 10 min at 4°C to release the cells from the alginate matrix. The cell suspension was centrifuged and total RNA was extracted from the cell pellet by RNeasy kit (Qiagen, Valencia, CA). Reverse transcriptase-polymerase chain reaction (RT-PCR)-ELISA was performed according to modifications of a previously reported procedure (Majumdar et al., 1998). RT-PCR was performed using total RNA as a template, oligonucleotide primers (Table 1), RNA PCR core kit (Perkin-Elmer, Norfolk, CT), and substituting the deoxy-nucleotides with digoxigenin-labeled nucleotides (Roche Biochemicals, Indianapolis, IN) to label the amplified products. ELISA was performed as recommended by the manufacturer (Roche Biochemicals). Briefly, the PCR product was denatured and allowed to hybridize in solution to a 5'-biotinylated probe at 37°C in a strepavidin-coated microtiter plate. The bound probe-PCR product was detected by an anti-digoxigenin peroxidase conjugate and by the use of the colorimetric substrate ABTS. The data for each untreated and TGF-β3–treated sample from each donor were normalized to β2-microglobulin. For each donor the induction of type II collagen expression by TGF-β3 was computed as fold increase over untreated. The result shown is the mean fold increase for 3 donors.

Immunohistochemistry

Alginate beads from cultures were washed with water and incubated in 100mM barium chloride for 10 min for irreversible polymerization. The beads were

TABLE 2. Incidence of CFU-Fs in the MNCs, $CD105^-$, and $CD105^-$ population of cells[1]

Procedure	Cell fractions			Fold
	MNCs	$CD105^-$	$CD105^-$	Enrichment
DAP (n = 8)	10.38 ± 1.60	38.75 ± 5.63	0.5 ± 0.26	3.73
SAP (n = 5)	11.06 ± 2.31	103.33 ± 11.97	0.60 ± 0.37	9.34

[1]MNCs, $CD105^-$ and $CD105^-$ cells were plated at 1×10^5 cells / well in 6-well plate in triplicate. The results represent the mean number of CFU-F ± SEM.

then washed with water again and fixed in 10% buffered formalin. Alginate sections were immunostained as previously reported (Sellers et al., 1997). Sections of alginate beads on glass slides were placed at 55°C for 45–60 min, washed in PBS, treated with trypsin for 10 min followed by 0.3% hydrogen peroxide (H_2O_2) for 5 min at room temperature and blocking solution for 30 min. For detection of type II collagen, goat anti-type II collagen antibody (Southern Biotechnology Associates, Birmingham, AL) was used at a 1:160 dilution overnight. Immunoreactivity was detected by incubating sections with biotinylated anti-goat antibody and horseradish peroxidase H reagents (Vector Laboratories, Burlingame, CA). Signal was developed by treating the sections with peroxidase substrate 3,3'-diaminobenzidine (DAB) and H_2O_2. Images were recorded on 35-mm slide film and multipanel figures were made with Photoshop (Adobe Systems, San Jose, CA). Experimental controls consisted of alginate sections stained with either the primary or the secondary antibody alone.

RESULTS

Isolation, culture expansion and analysis of $CD105^+$ cells

Isolation and analysis of $CD105^+$ cells. To isolate multipotential stromal cells from human bone marrow, we developed a procedure which employed a magnet-based cell separation column for the positive selection of cells recognized by antibody conjugated to microbeads. As we had little information on the number of CD105 molecules on the surface of stromal cells, the DAP procedure was developed so that the two antibodies together would result in an amplified signal, aiding in the isolation of the stromal cells. The SAP procedure was developed to compare the efficiency between the procedures. Stromal cells isolated from multiple human bone marrow samples by the DAP and the SAP for $CD105^+$ cell selection represented 3 ± 0.9% (n = 7) and 1.76 ± 0.33% (n = 8) of the MNCs, respectively. CFU-F assay performed on isolated cells using both the DAP and the SAP showed over 3-fold and 9-fold (Table 2) greater incidence of CFU-Fs respectively in the $CD105^+$ population compared to that in the unseparated MNCs. The cells in the colonies displayed a fibroblastic morphology in monolayer culture and maintained similar phenotype with passage. In most donors there was no detectable CFU-F in the $CD105^-$ cell population. Therefore, both the DAP and SAP procedures were able to achieve $CD105^+$ cell recovery of nearly 100% from the ficoll-separated MNCs.

Culture expansion and analysis of $CD105^+$ cells. To establish an optimum culture condition for the attachment and expansion of $CD105^+$ cells without undergoing differentiation, various combinations of base media with different percent of fetal bovine serum were tested in the CFU-F assay. Alpha-MEM supplemented with 10% fetal bovine serum and 1% antimycotic-antibiotic showed a significant increase in CFU-Fs over DMEM-based medium and there was no significant difference in CFU-Fs between media supplemented with 10% or 20% fetal bovine serum (data not shown). The cells in the colony were phenotypically fibroblastic and proliferated in a distinct whorl pattern (Fig. 1). After 14 days in culture, the colonies were pooled and the cells were designated primary cultures (p0). Use of the DAP resulted in $1.56 \pm 0.17 \times 10^6$ (n = 6) p0 cells for every 1×10^6 $CD105^-$ cells originally plated, while use of the SAP resulted in $4.44 \pm 0.44 \times 10^6$ (n = 7) for every 1×10^6 $CD105^-$ cells originally plated. Primary cells analyzed by flow cytometry showed approximately 98% of the cells to be positive for CD105 cell surface molecule and negative for leukocyte common antigen, CD45 (Fig. 2). This observation showed that the expanded cells were a homogenous population. For further expansion, cells were replated at 1×10^6 per 185/cm^2 flasks as passage 1 cells (p1). Cells recovered at confluence between 6–7 days in culture were between 3–5 $\times 10^6$ cells per flask and this

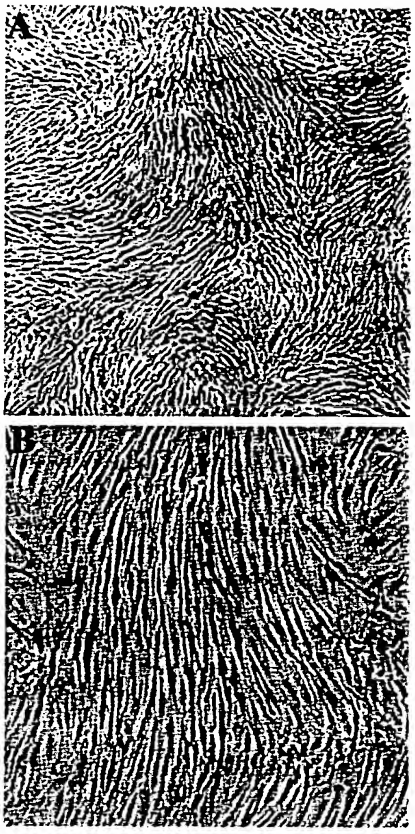


Fig. 1. Morphology of primary and passage 1 cultures of $CD105^+$ cells. The cells were isolated from bone marrow by using microbeads coupled-antibody against CD105 and plated in media. The cultures were maintained for 14 days and the cells were termed primary. The cells were trypsinized and replated as passage 1 cells. Photomicrographs are representative of primary (**A**, × 40) and passage 1 (**B**, × 100) cultures as shown above.

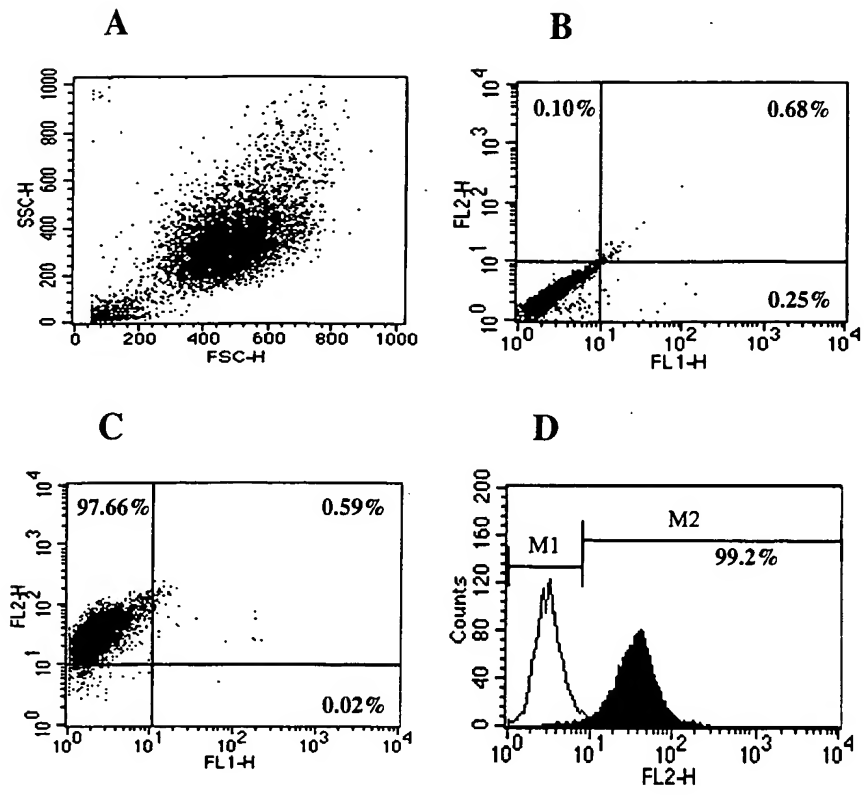


Fig. 2. Flow cytometry analysis of cultured CD105⁻ cells with monoclonal antibodies. The cells were isolated from bone marrow by using microbeads coupled-antibody against CD105, plated in media and the culture was maintained for 14 days. The cells were trypsinized and aliquots of the pooled cells were stained with monoclonal antibodies and analyzed by flow cytometry. **A:** Unstained cells to show the forward and side scatter. **B:** Cells stained with mouse isotype antibodies. **C:** Cells stained with antibodies against human leukocyte common antigen CD45 (FL1, CD45-FITC) and endoglin CD105 (FL-2, CD105-PE). **D:** Cells stained with mouse isotype (M1, mIgG-PE) antibody and endoglin CD105 (M2, CD105-PE). The percent (%) of cells in the quadrants and margins were determined. These data are representative of analysis performed on cells isolated from multiple donors.

rate of expansion was maintained for the next 4 passages.

Cell surface phenotype of CD105⁺ cells. To define the phenotype of CD105⁺ cells, we analyzed the cell surface molecules expressed by primary and passaged cells of three different donors by means of flow cytometry. We chose a selected group of molecules have been shown to be either present or absent on stromal and mesenchymal cells derived from human bone marrow (Simmons, 1994; Pittenger et al., 1999). The molecules were grouped into peptidases (CD10 and CD13), integrins (CD11a, CD18, 29, CD49b, CD49d, CD49e, CD49f), matrix receptors (CD31, CD54, CD62l, CD62p, CD106), and others like hyaluronate (CD44), transferrin receptor (CD71), thy-1 (CD90), and endoglin (CD105). The results (Table 3) show that CD105⁺ cells maintained the expression of CD105 with culture expansion. Surface molecules expressed by greater than 50% of the cells include CD13, CD29, CD44, CD49b, CD49e, CD54, CD71, and CD90. Surface molecules expressed by 5–50% of the cells include CD10, CD11a (p0), CD49f, and CD62p (p0). Molecules expressed by less than 5% of the cells include CD11a (p5), CD18, CD31, CD62l, and CD62p (p5). After five passages, expression of most of the cell surface molecules were similar to primary cells (p0), which suggests that the cells did not undergo phenotypic changes with passage in culture. The expression of these molecules are similar to stromal precursor cells (Simmons, 1994) and mesenchymal stem cells (Pittenger et al., 1999). We also observed donor to donor variability in the expression of alpha 5 and 6 integrins, and in the expression of ICAM-1 between primary and passaged cells. There was no significant difference in the expression of cell surface molecules between trypsin and non-trypsin detached cells.

TABLE 3. Analysis of cell surface molecules of CD105⁻ cells (n = 3)[1]

Cell surface molecule	Primary (p0, day 14)	Passage 5 (p5)
CD10 Metalloproteinase	34.3 ± 2.87	45.8 ± 7.2
CD11a Alpha L Integrin	8.3 ± 2.0	4.6 ± 0.6
CD11c Leucocyte Adhesion Receptor	5.0 ± 1.1	4.4 ± 0.4
CD13 Metalloproteinase	99.4 ± 0.3	99.7 ± 0.3
CD18 Beta 2 Integrin	2.4 ± 0.1	3.5 ± 0.1
CD29 Beta 1 Integrin	87.5 ± 3.6	90.7 ± 3.6
CD31 PECAM-1	4.6 ± 0.4	4.8 ± 0.2
CD44 Hyaluronate	99.3 ± 0.4	98.3 ± 1.1
CD49b Alpha 2 Integrin	80.6 ± 0.6	82.2 ± 7.9
CD49e Alpha 5 Integrin	72.5 ± 10.9	53.8 ± 0.8
CD49f Alpha 6 Integrin	47.7 ± 15.9	30.8 ± 11.1
CD54 ICAM-1	65.9 ± 2.5	80.1 ± 2.9
CD62l L-Selectin	4.7 ± 0.1	3.7 ± 0.9
CD62p P-Selectin	10.9 ± 2.9	4.6 ± 0.8
CD71 Transferrin Receptor	71.5 ± 3.6	82.8 ± 5.8
CD90 Thy-1	99.5 ± 0.3	100.0
CD105 Endoglin	99.0 ± 0.4	97.8 ± 4.0

[1]The CD105⁻ cells were detached by trypsin treatment, labeled with antibody against the molecules as described in text, and analyzed by flow cytometry. The results are expressed as mean ± SEM.

To analyze the expression of TGF-β receptors by the CD105⁺ cells, reverse transcriptase-polymerase chain reaction (RT-PCR)-ELISA (Majumdar et al., 1998) was performed with total RNA isolated from 20 individual clones from three donors. Results (data not shown) showed that all the 20 clones expressed both the type I

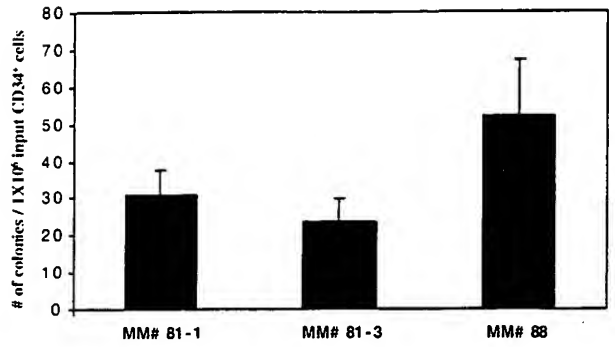


Fig. 3. The ability to support hematopoiesis in vitro by CD105$^+$ cells from 3 donors. The cells were isolated from 3 bone marrow donors (MM# 81-1, 81-3, and 88) by using microbead coupled-antibody against CD105 and plated in media. The cultures were maintained for 14 days and the cells were trypsinized and replated as passage 1 cells. On reaching 80–90% of confluence the cells were irradiated and cocultured for 5 weeks with CD34$^+$ hematopoietic progenitor cells. Both the nonadherent and the adherent cell populations were pooled and cultured in methylcellulose for 2 weeks and the hematopoietic colonies were counted. The bars represent the mean (± SEM) colony numbers from three individual wells.

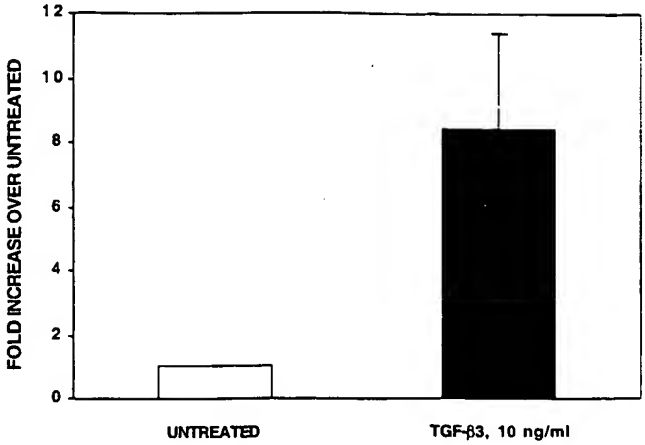


Fig. 4. Type II collagen mRNA expression by passaged CD105$^+$ cells in alginate cultures. Culture-expanded passage 3 cells were resuspended in alginate and cultured in a serum-free media (untreated) supplemented with TGF-β3 for 3 weeks. RT-PCR ELISA for type II collagen was performed on RNA extracted from the alginate cells. The bars represent the mean (± SEM) from 3 donors.

and II TGF-β receptors which further provided significant evidence to the homogeneity of the CD105$^+$ cells.

Long term bone marrow culture

One of the characteristics of bone marrow-derived stromal cells is their ability to support hematopoiesis in vitro (Dexter et al., 1977). To demonstrate that CD105$^+$ cells were able to support hematopoiesis, LTBMCs were established with irradiated CD105$^+$ cells from 3 donors and hematopoietic progenitor CD34$^+$ cells. During the 5 weeks of co-culture, cobblestone areas representing hematopoietic progenitor cell proliferation and differentiation were observed (data not shown). When plated in methylcellulose, cells derived from the co-culture resulted in myeloid hematopoietic colony formation (Fig. 3). These results showed that the culture expanded CD105$^+$ cells were able to maintain their stromal phenotype with passage and thus able to support hematopoiesis. The difference seen in the number of hematopoietic colonies that formed in methylcellulose indicated donor to donor variability and were comparable to previously reported results (Majumdar et al., 1998). Experimental controls of LTBMC of CD34$^+$ cells in the absence of CD105$^+$ cells did not result in any colony formation.

Expression and synthesis of type II collagen by CD105$^+$ cells

Mesenchymal stem and progenitor cells have been cultured as aggregates (Johnstone et al., 1998; Mackay et al., 1998; Yoo et al., 1998) to provide a 3-dimensional environment which promotes differentiation along the chondrogenic lineage with the expression of type II collagen, a definitive marker of the chondrogenic phenotype. We modified the alginate culture conditions to make it conducive for the CD105$^+$ cells to differentiate similarly. To our knowledge this is the first report of the culture of stromal cells in alginate for chondrogenic differentiation. RT-PCR ELISA performed on RNA extracted from cells of 3 donors cultured in alginate beads at day 21 showed about a 9-fold increase in type II collagen mRNA expression in cells treated with TGF-β3 over untreated cells (Fig. 4). Immunostaining of the alginate bead sections demonstrated synthesis of type II collagen protein (Fig. 5) by the cells treated with TGF-β3. Type II collagen protein was also present in the intercellular region due to the secretion of the protein by the differentiating cells and subsequent entrapment in the alginate matrix. Alginate sections stained with either the primary or the secondary antibody did not show any immunoreactivity. CD105$^-$ cells did not express type II collagen in monolayer cultures (data not shown).

DISCUSSION

The relationship between bone marrow stromal cells and repair of articular cartilage is well established from in vivo and in vitro studies (Suh et al., 1997; O'Driscoll, 1998). Repair of damaged articular cartilage requires the mobilization and differentiation of these cells at the damaged site, although the newly formed tissue in most cases was not articular cartilage (Buckwalter et al., 1990; Buckwalter and Mankin, 1998b). One possible reason may be that the factors or the signals present during articular cartilage development are no longer present in the adult. The complex in vivo environment makes it difficult for the identification of the differentiating factors that are important in the transformation of progenitor cells into chondrocytes. Therefore, despite the enhanced interest in the field of cartilage regeneration, the mechanism of chondrogenesis is still poorly understood. To better understand the process of chondrogenesis and to identify the factors involved, it is essential to develop an efficient and reproducible procedure to isolate the target cells and also an in vitro culture system that promotes chondrogenic differentiation of these cells. In the present study, we describe a procedure to isolate multipotential stromal cells based on the presence of a cell surface molecule and an in vitro system to differentiate these

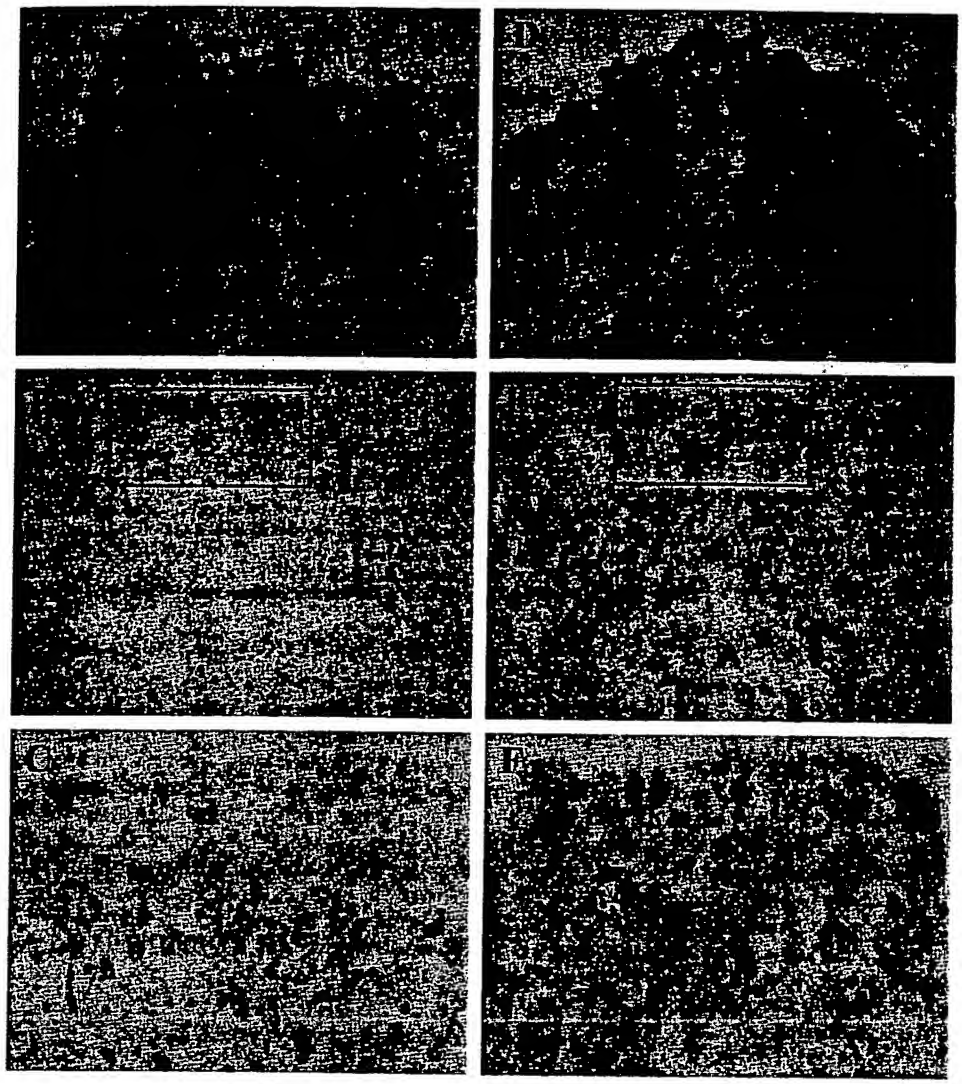


Fig. 5. Alginate culture induced chondrogenesis in passaged CD105$^+$ cells. Culture-expanded passage 3 cells were resuspended in alginate and cultured in a serum-free media (**A–C**) or supplemented with TGF-β3 (**D–F**) for 3 weeks. The beads were then irreversibly polymerized and sections were stained for the detection of type II collagen protein. A and D represent sections stained with hematoxylin and eosin. B, C, E, and F represent sections immunostained for type II collagen. Enlargements of B and E are shown in C and F, respectively.

cells along the chondrogenic lineage. We also demonstrate the ability of these cells to support hematopoiesis, an important biological function of stromal cells which confirms their multipotential characteristic.

Bone marrow stroma is comprised of a heterogeneous group of cells both of the hematopoietic and non-hematopoietic lineages (Dorshkind, 1990). Therefore, isolation of the stromal cells which represents a small percentage of the total marrow cells (Gronthos and Simmons, 1995; Pittenger et al., 1999) requires both phenotypic and functional information about these multipotential cells. Stromal cells including mesenchymal stem and progenitor cells have the ability to adhere to tissue culture surfaces and form fibroblastic colonies, CFU-Fs (Haynesworth et al., 1992; Gronthos and Simmons, 1995; Kadiyala et al., 1997; Fortier et al., 1998; Pittenger et al., 1999). These cells respond to TGF-β in vitro for chondrogenic progression (Johnstone et al., 1998; Mackay et al., 1998; Pittenger et al., 1999). Endoglin (CD105), a member of the TGF-β receptor family, has been shown to be expressed by these cells (Robledo et al., 1996). We utilized this information to formulate our stromal cell isolation strategy. To our knowledge this study is the first report in which isolation of multipotential stromal cells was based on the presence of CD105, a well characterized cell surface molecule with biological function (Li et al., 1999).

In our stromal cell isolation studies, we developed DAP and SAP. Although DAP was reproducible, we were unable to achieve the efficiency reported in a previous study (Gronthos and Simmons, 1995). One possible explanation was the formation of cell clumps during the prolonged antibody incubation steps which may have resulted in lower cell viability and also may have led to loss of adherence. Results from flow cytometry, LTBMC, and alginate culture studies confirmed that DAP cells were multipotential. With the availability of primary antibody-conjugated microbeads, we were able to show considerable increase in cell plating ability and also attained the efficiency, previously reported. These results are significant because the CFU-F assays were performed without the use of cell attachment enhancers like media supplemented with 20% fetal bovine serum and fibronectin coated plates as

reported by Gronthos and Simmons (1995). The higher efficiency of SAP may be attributed to the absence of cell clumps due to brief antibody incubation step and increased cell viability. SAP also resulted in a 2.8-fold increase in cell recovery over DAP at the end of primary culture. There were no differences between the SAP- and the DAP-isolated cells in the phenotypic and biological functions as observed from flow cytometry, LTBMC, and alginate culture studies (data not shown).

Stromal cells have been shown to have a unique expression profile of cell surface molecules (Dorshkind, 1990; Deryugina and Muller-Sieburg, 1993; Simmons, 1994; Pittenger et al., 1999). We performed an extensive study on the expression of cell surface molecules on the $CD105^+$ cells, both as primary and multiple passaged cells to analyze the changes in expression profile with cell expansion. The results (Table 2) show that the $CD105^+$ cells have a cell surface expression profile similar to mesenchymal stem cells (Pittenger et al., 1999) and stromal precursor cells (Simmons, 1994). The expanded cells also showed a similar expression profile suggesting the maintenance of the undifferentiated characteristic. The expression of this array of molecules by the $CD105^+$ cells also suggests the potential to perform multiple functions. The peptidases (CD10 and CD13) expressed by the $CD105^+$ cells may function in modifying other cell surface proteins on its surface or on neighboring cells. The expression of integrins and adhesion molecules may help in the interaction between stromal and hematopoietic cells as well as with the extracellular matrix which is an important component of the bone marrow stroma (Hynes, 1992). Endoglin is an important member of the type III TGF-β receptor which binds to TGF-β superfamily and interacts with their receptors, and likely regulates TGF-β signaling. It is an essential component for angiogenesis during embryonic development (Li et al., 1999). Loss-of-function in the human endoglin gene causes hereditary hemorrhagic telangiectasia (Guttmacher et al., 1995). Little is known about the role of endoglin during bone and cartilage development. While cartilage is an avascular tissue, on the contrary bone is highly vascular, therefore the role of endoglin during endochondral ossification may provide us with valuable information about bone and cartilage development and the fate of the $CD105^+$ cells.

The $CD105^+$ cells did not express markers of the hematopoietic lineage, including the lipopolysaccharide receptor CD14, CD34, and the leukocyte common antigen CD45. The $CD105^+$ cells eluted from the magnetic column when incubated with antibody raised against Stro-1 resulted in over 60% of the cell expressing the molecule (data not shown), which correlated well with previous studies (Gronthos and Simmons, 1995).

Mesenchymal stem cells have potential to differentiate along multiple connective tissue lineages including adipogenic, osteogenic and chondrogenic (Pittenger et al., 1999) and also support hematopoiesis in vitro (Majumdar et al., 1998), a hallmark of bone marrow-derived stromal cells. To evaluate the multipotential characteristics of the $CD105^+$ cells, we examined the ability of these cells to differentiate along one of the connective tissue lineages, the chondrogenic lineage and another distinct phenotype, its stromal characteristic to support hematopoiesis in vitro. Expression of type II collagen is a phenotype of articular chondrocytes (Binette et al., 1998), as well as in vitro aggregate cultures of mesenchymal stem and progenitor cells undergoing chondrogenesis in response to TGF-β (Johnstone et al., 1998; Pittenger et al., 1999). For type II collagen expression studies, $CD105^+$ cells were cultured in alginate instead of the cell aggregate culture system. Cells from the alginate culture system were easily recoverable by dissolving the alginate matrix in citrate (Mok et al., 1994) and provided further opportunities to evaluate the differentiated cells which was not possible for cells in aggregate cultures. The results from alginate culture studies showed that the $CD105^+$ cells had the ability to express type II collagen in response to TGF-β3 and this held true for cells isolated from multiple donors and multiple passages (data not shown). Like mesenchymal stem cells (Majumdar et al., 1998), $CD105^+$ cells were able to support hematopoiesis in vitro, which designated a true bone marrow stromal phenotype to these cells.

In this study we showed the development of an efficient and reproducible procedure to isolate multipotential bone marrow stromal cells and also introduced a culture system that allowed the differentiation of these cells towards the chondrogenic lineage. The combination of these two procedures will provide useful information in defining the process of chondrogenesis.

ACKNOWLEDGEMENTS

The authors thank Drs. Glenn Larson, Katherine Turner, Renwen Zhang, Hui-Min Chen and Sonya Glasson for helpful discussion and for reviewing the manuscript; Hemchand Sookdeo and the DNA synthesis group for synthesizing the oligonucleotide primers.

LITERATURE CITED

Bently SA. 1982. Bone marrow connective tissue and the hematopoietic microenvironment. Br J Haematol 50:1.

Binette F, McQuaid DP, Haudenschild DR, Yaeger PC, McPherson JM, Tubo R. 1998. Expression of a stable articular cartilage phenotype without evidence of hypertrophy by adult human articular chondrocytes in vitro. J Orthop Res 16:207–216.

Buckwalter, J, Mankin H. 1998a. Atricular cartilage repair and transplantation. Arthritis Rheum 42:1331–1342.

Buckwalter JA, Mankin HJ. 1998b. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. In: Dilworth Cannon Jr. W, editor. AAOS instructional courses lectures. Rosemont, IL: American Association of Orthopaedic Surgeons. Vol. 47: 487–504.

Buckwalter JA, Rosenberg L, Hunziker EB. 1990. Articular cartilage: composition, structure, response to injury and methods of facilitating repair. In: Ewing JW, editor. Articular cartilage and knee joint function: basic science and arthroscopy. New York: Raven Press New York.

Deryugina EI. Muller-Sieburg CE. 1993. Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development? Crit Rev Immunol 13:115–150.

Dexter TM, Allen TD, Lajtha LG. 1977. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335–344.

Dorshkind K. 1990. Regulation of hemopoiesis by bone marrow stromal cells and their products. Annu Rev Immunol 8:111–137.

Ferrari G, Angelis GC, Coletta M, Paolucci E, Stornaiuolo A, Mavilio F. 1998. Muscle regeration by bone marrow-derived myogenic progenitors. Science 279:1528–1530.

Fortier LA, Nixon AJ, Williams J, Cable CS. 1998. Isolation and chondrogenic differentiation of equine bone marrow-derived mesenchymal stem cells. Am J Vet Res 59:1182–1187.

Gimble JM, Robinson CE, Wu X, Kelly KA. 1996. The function of adipocyte in the bone marrow stroma: an update. Bone 19:421–428.

Gronthos S, Simmons PJ. 1995. The growth factor requirements of Stro-1-positive human stromal precursors under serum-deprived conditions in vitro. Blood 85:929–940.

Guttmacher AE, Marchuk DA, White RI Jr. 1995. Hereditary hemorrhagic telangiectasia [see comments]. N Engl J Med 333:918–924.

Haynesworth SE, Goshima J, Goldberg VM, Calplan AI. 1992. Characterization of cells with osteogenic potential from the human bone marrow. Bone 13:81–95.

Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. 1998. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272.

Kadiyala S, Young RG, Thiede MA, Bruder SP. 1997. Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. Cell Transplant 6:125–134.

Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP. 1999. Defective angiogenesis in mice lacking endoglin. Science 284:1534–1537.

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. 1998. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415–428.

Majumdar M, Thiede M, Mosca J, Moorman M, Gerson S. 1998. Phenotypic and functional comparison of cultures of bone marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 176:57–66.

Mok S, Masuda K, Hauselmann H, Aydelotte M, Thonar E. 1994. Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. J Biol Chem 269:33021–33027.

O'Driscoll SW. 1998. The healing and regeneration of articular cartilage. J Bone Joint Surg Am 80:1795–1812.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DA. 1999. Multilineage potential of adult mesenchymal stem cells. Science 284:143–147.

Robledo M, Hidalgo A, Lastres P, Arroyo A, Bernabeu C, Sanchez-Madrid F, Teixido J. 1996. Characterization of TGF-β1-binding proteins on human bone marrow stromal cells. Br J Haematol 93:507–514.

Sellers R, Peluso D, Morris E. 1997. The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. J Bone Joint Surg Am 79:1452–1463.

Sellers R, Zhang R, Glasson S, Kim H, Peluso D, D'Augusta D, Beckwith K, Morris E. 2000. Repair of articular cartilage one year after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). J Bone Joint Surg Am 82A:151–160.

Simmons PJ, Gronthos S, Zannettino A, Ohta S, Graves S. 1994. Isolation, Characterization and functional activity of bone marrow stromal progenitors in hematopoiesis. In: Worthington-White D, Gross S, Gee A, editors. Advances in bone marrow purging and processing: fourth international symposium. I. New York: Wiley-Liss. 271–280.

Suh JK, Aoren A, Muzzonigro TS, Disilvestro M, Fu FH. 1997. Injury and repair of articular cartilage: related scientific issues. Op Tech Ortho 7:270–278.

Tavassoli M, Friedstein A. 1983. Hematopoietic stromal microenvironment. Am J Hematol 15:195.

Weiss, L. 1995. Functional organization of hematopoietic tissues. In: Hoffman R, Benz E, Shattil S, Furie B, Zcohen H, Silverstein L, editors. Hematology: basic principles and practice. New York: Churchill Livingston. 193–207.

Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B. 1998. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am 80:1745–1757.

Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. 1998. Use of mesenchymal stem cells in a collagen matrix for achilles tendon repair. J Orthop Res 16:406–413.

CERTIFICATE UNDER 37 CFR § 1.10 OF MAILING BY "EXPRESS MAIL"

EV 618318728 US	4/5/05
USPS Express Mail Label Number	Date of Deposit

I hereby certify that this correspondence is being deposited with the United States Postal Services "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on the date indicated above and is addressed to the Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

By: Mona Bieler

Mona Bieler